AD_____

Award Number: DAMD17-94-J-4303

TITLE: Mechanism of Integrim-Mediated Growth Control in Normal,
Transformed, and Neoplastic Breast Cells

PRINCIPAL INVESTIGATOR: Elizabeth Wayner, Ph.D.
                        Richard Tamura, Ph.D.

CONTRACTING ORGANIZATION: Seattle Biomedical Research Institute
                          Seattle, Washington 98109-1651

REPORT DATE: October 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
              Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
                        distribution unlimited

The views, opinions and/or findings contained in this report are those
of the author(s) and should not be construed as an official Department
of the Army position, policy or decision unless so designated by other
documentation.

20000822 060

REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 074-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE October 1999	3. REPORT TYPE AND DATES COVERED Final (30 Sep 94 - 29 Sep 99)	
4. TITLE AND SUBTITLE Mechanism of Integrim-Mediated Growth Control in Normal, Transformed, and Neoplastic Breast Cells			**5. FUNDING NUMBERS** DAMD17-94-J-4303
6. AUTHOR(S) Elizabeth Wayner, Ph.D. Richard Tamura, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Seattle Biomedical Research Institute Seattle, Washington 98109 **e-mail:** rtamura@u.washington.edu			**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited	**12b. DISTRIBUTION CODE**

13. ABSTRACT (Maximum 200 Words)

Anchorage-dependent cell growth by normal cells and the loss of this dependence during tumorigenesis is a phenomenon that has been recognized for many years. The primary cell adhesion receptors that mediate binding to extracellular matrix proteins are integrins Our data suggest that $\alpha3\beta1$ and $\alpha6\beta4$ are the primary integrins responsible for mediating the G1/S transition in normal mammary epithelial cells and that these mechanisms may be defective in metastatic cancer cells. Other integrins also appear to influence components of the cell cycle regulatory machinery. For example, binding of GRGDS peptides to integrins ($\alpha v\beta5$, $\alpha v\beta3$, $\alpha5\beta1$) on both normal and cancer cells appears to regulate the activities of cdc2 kinase and cyclinA-associated kinases. Cancer cells appear to have numerous defects in both cell adhesion components ($\alpha6\beta4$, E-cadherin, laminin-5) and cell cycle regulatory components (cyclin D2, p21) which may contribute to the adhesion-independent phenotype. A difference in expression of the alternatively-spliced forms of the integrin $\alpha6$, $\alpha6A$ and $\alpha6B$, suggests that this marker may have diagnostic or prognostic value. Further study of the significance of these defects may lead to the development of novel therapeutics for the treatment or cure of breast cancer.

14. SUBJECT TERMS Adhesion, Ligand-Receptor Interactions, Surface Receptors, Transformation, Growth, Signalling, Humans, Anatomical Samples, Breast Cancer, Integrins, Cell Proliferation			**15. NUMBER OF PAGES** 21
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	**18. SECURITY CLASSIFICATION OF THIS PAGE** Unclassified	**19. SECURITY CLASSIFICATION OF ABSTRACT** Unclassified	**20. LIMITATION OF ABSTRACT** Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

_✓ RWT For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_✓ RWT In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_✓ RWT In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature   Date   11/1/99

RESEARCH GRANT DAMD 17-94-J-4303
PRINCIPAL INVESTIGATORS: RICHARD N. TAMURA
AND ELIZABETH A. WAYNER

TABLE OF CONTENTS

INTRODUCTION

Anchorage-dependent cell growth by normal cells and the loss of this dependence during tumorigenesis is a phenomenon that has been recognized for many years. The mechanisms underlying these processes have only recently begun to be elucidated. The primary cell adhesion receptors that mediate binding to extracellular matrix proteins are integrins (Hynes, 1992). Integrins provide not only a structural means of cell anchorage but also a means of transmitting signals regulating cell proliferation, differentiation, gene expression and protein function (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999).

The author of this grant, Dr. Banu Symington, proposed originally to "characterize growth signals elicited by different FNR ligands in non-transformed breast cells, and to evaluate how these signals differ for neoplastic cells."

The original specific aims were:
Aim 1) Identify the mechanism of FNR-mediated growth stimulation in normal breast cells and non-transformed breast cell lines.

Aim 2) Examine the effect of GRGDS on growth and cdc2 activation in transformed human breast cell lines and identify the cause(s) underlying abnormal growth responses to GRGDS.

Aim 3) Examine the effect of GRGDS on growth and cdc2 activation in freshly isolated neoplastic breast cells and identify the cause(s) underlying abnormal growth responses to GRGDS.

Results obtained pursuing these objectives suggested that it would be appropriate to modify the statement of work and specific aims. In the 1997 annual report, the following changes were made:

Statement of Work:
The purpose of this project is to determine the role of integrins in regulating the proliferation of normal and neoplastic mammary epithelial cells.

Specific Aims:
Aim 1: Determine whether all integrins or a subset of integrins are involved in regulating normal mammary epithelial cell proliferation.

Aim 2: Determine the mechanisms of integrin-mediated cell cycle regulation.

Aim 3: Examine carcinoma cells for possible defects in these mechanisms.

BODY

Materials and Methods

The various materials and methods utilized in this study have been described in previous annual reports.

Results and Discussion

The original purpose of this project was to determine how signals transduced via the fibronectin receptor, integrin $\alpha5\beta1$, regulated the formation of cdk/cyclin complexes in neoplastic and normal breast cells. This tenet was based upon observations that suggested that binding of the fibronectin peptide ligand, GRGDS, to the integrin $\alpha5\beta1$ regulated cell proliferation through modulation of cdc2/cyclinA complexes in partially transformed but not fully transformed epithelial cells (Symington, 1992; Symington, 1995). These initial studies compared two breast cancer cell lines (HBL 100 and BT20) as well as normal human epidermal keratinocytes with two virally transformed keratinocyte cell lines.

The original specific aims were:
Aim 1) Identify the mechanism of FNR-mediated growth stimulation in normal breast cells and non-transformed breast cell lines.

Aim 2) Examine the effect of GRGDS on growth and cdc2 activation in transformed human breast cell lines and identify the cause(s) underlying abnormal growth responses to GRGDS.

Aim 3) Examine the effect of GRGDS on growth and cdc2 activation in freshly isolated neoplastic breast cells and identify the cause(s) underlying abnormal growth responses to GRGDS.

We expanded Dr. Symington's GRGDS peptide studies to normal human mammary epithelial cells (HMEC) and to mammary carcinoma cell lines that appeared to differ in tumorigenic or metastatic potential. The GRGDS peptides affected cdc2 kinase or cyclinA associated kinase activities in normal HMEC as well as a number of breast cancer cell lines (Fig 1 and Fig 2, 1996 annual report). However, the changes in cdc2 kinase activities usually did not correspond with concomitant changes in cyclinA associated kinase activity suggesting that complexes other than cdc2-cyclinA were affected by the RGD peptides. Also, the changes in the activities of these cell cycle kinases did not correlate with the degree of transformation of the cells.

These studies were complicated by the fact that other integrins, mainly $\alpha v\beta3$ and $\alpha v\beta5$, also bind RGD peptides. We therefore characterized the expression of integrins on normal HMEC and the various cell lines by FACS analysis (Table I, 1996 annual report). It should be noted that the data for integrin $\beta4$ expression in Table I, 1996 annual report, is inaccurate. We did not realize that the antibodies used for the $\beta4$ FACS did not react with the extracellular domain of $\beta4$ until they were further characterized at a much later date. The regulation of cdc2 kinase activities or cyclinA associated kinase activities by RGD peptides could not be explained by the pattern or level of expression $\alpha5\beta1$, $\alpha v\beta3$, or $\alpha v\beta5$ (1997 annual report, Table I).

The expression of integrins, ECM proteins, and cell cycle components were analyzed by immunohistochemistry of normal and tumor breast tissue. Incubation with the anti-integrin α5 monoclonal antibody, P1D6, suggested that α5β1 was not expressed by either normal mammary epithelial cells or on tumor cells (Fig 3, 1996 annual report). Since P1D6 readily stained fibroblasts in the stroma, we did not test other monoclonal antibodies to the integrin α5 subunit. However, we probably should have utilized other monoclonal antibodies to α5 to ensure that our results were not epitope dependent. The most prominent integrin expressed by both normal mammary epithelial cells and tumor cells was the integrin α3β1 (Fig 4, 1996 annual report).

Based up these results and observations, it seemed appropriate to modify the statement of work and specific aims of this project. In the 1997 annual report, the following changes were made:

Statement of Work:
The purpose of this project is to determine the role of integrins in regulating the proliferation of normal and neoplastic mammary epithelial cells.

Specific Aims:
Aim 1: Determine whether all integrins or a subset of integrins are involved in regulating normal mammary epithelial cell proliferation.

Aim 2: Determine the mechanisms of integrin-mediated cell cycle regulation.

Aim 3: Examine carcinoma cells for possible defects in these mechanisms.

Aim 1: Determine whether all integrins or a subset of integrins are involved in regulating normal mammary epithelial cell proliferation.

In addition to the cdc2 kinase and cyclinA associated kinase experiments, we conducted integrin clustering experiments on cultures of normal HMEC that were synchronized to G1 by growth factor deprivation. The effects of integrin clustering on cell proliferation were evaluated by Western blot analyses of Rb phosphorylation. Clustering with antibodies to integrin β1, α3, β4, or αv subunits appeared to enhance the rate of Rb phosphorylation over GF alone (Fig 3, 1997 annual report).

The role of integrins in regulating cell proliferation was also evaluated by plating cells synchronized by serum/growth factor deprivation onto wells coated with ECM proteins or with monoclonal antibodies to specific integrin subunits. The number of cells present in each well was assayed 2-5 days after plating. Laminin-5 and Type I collagen but not fibronectin appeared to promote the growth of synchronized HMEC (Fig1, 1997 annual report). Thus, taken together, the RGD peptide binding, integrin clustering, and cell growth assays suggested that multiple integrins were capable of regulating the cell cycle machinery.

Normal HMEC were very troublesome to work with due to the limited lifespan and slow growth rate of these cells. Delays were often encountered expanding cultures to the requisite numbers for an experiment and,

sometimes, the cultures died or underwent terminal differentiation before or during an experiment. We therefore obtained the MCF12A cell line from the ATCC. This cell line was originally developed at the Karmanos Cancer Institute (Detroit, MI) and was characterized as a normal mammary epithelial cell line.

Cells synchronized by serum deprivation were plated onto wells coated with monoclonal antibodies to integrins in media containing reduced levels of growth factors (0.1% of the standard complete medium). Cell proliferation was stimulated by antibodies to integrin subunits $\beta 1$, $\alpha 3$, $\beta 4$, $\alpha 6$, and αV in the normal mammary epithelial cell line MCF12A but not in the metastatic breast carcinoma cell line MDA-MB-435 (Fig 1 and Fig 2, 1998 annual report). This difference was observed over a broad range of growth factors concentrations (Fig 1, Fig 2).
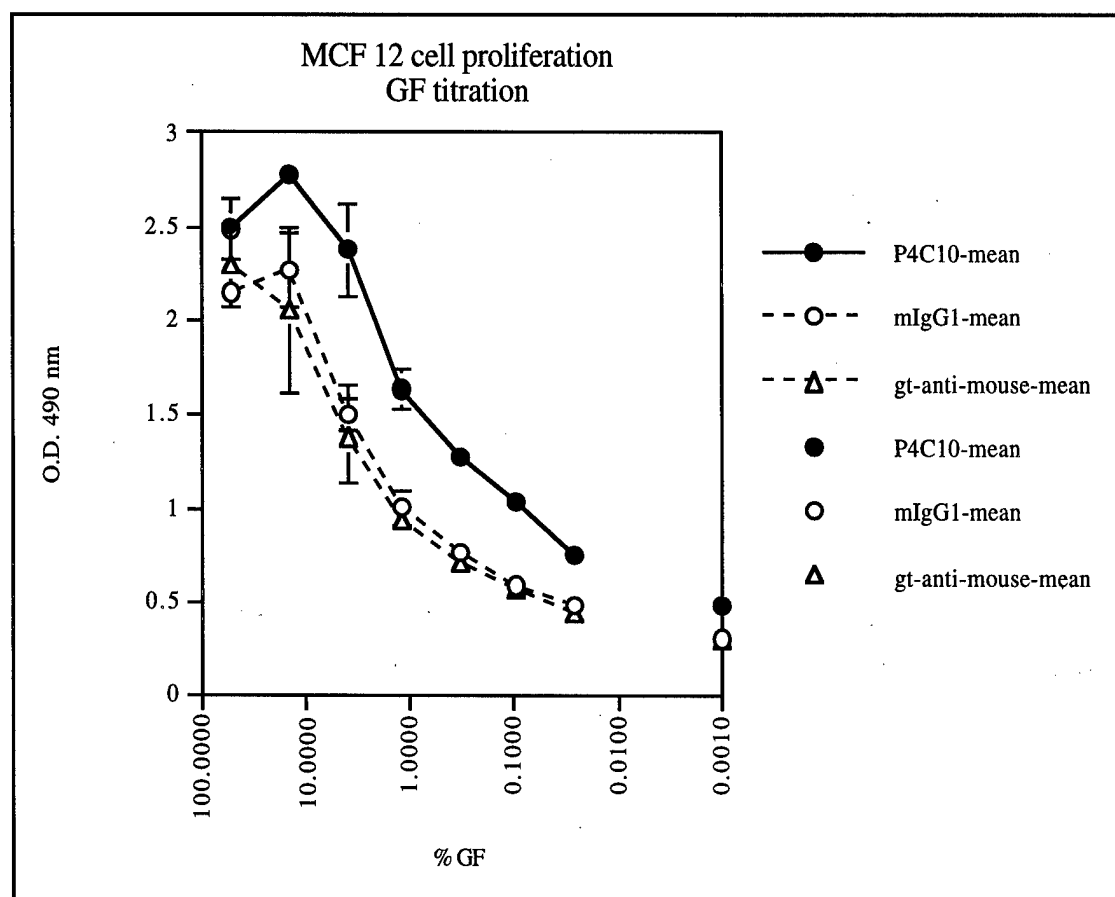

Fig 1. Stimulation of MCF12A cell proliferation by anti-integrin antibodies, growth factor (GF) titration. Synchronized cultures of MCF12A cells were plated onto 96 well microtiter plates coated with anti-integrin antibodies in medium containing serial dilution of GF. Cell proliferation was assayed 4 days later.
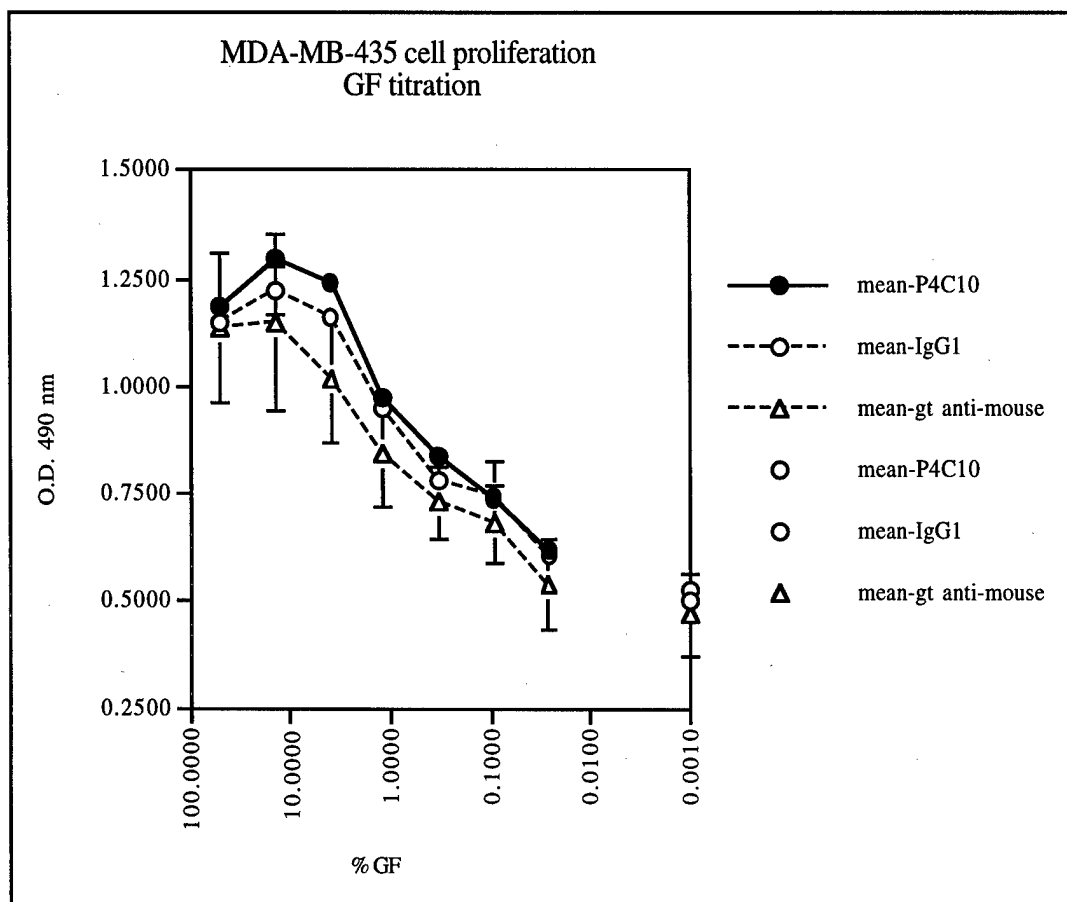
Fig 2. Stimulation of MDA-MB-435 cell proliferation by anti-integrin β1 antibodies, GF titration. Synchronized cultures of MDA-MB-435 cells were plated onto 96 well microtiter plates coated with anti-integrin β1antibodies in medium containing serial dilutions of GF. Cell proliferation was assayed 4 days later.

Integrin modulation of cell proliferation is dependent upon, and proportionate to, the level of growth factors (Fig 1). This suggests that integrin engagement is acting upon the normal growth factor signaling pathways.

Aim 2: Determine the mechanisms of integrin-mediated cell cycle regulation.

One possible mechanism of integrin regulation of cell proliferation is through the association and activation of growth factor receptors with activated integrin clusters as suggested by Plopper et al (Plopper et al., 1995) and Miyamoto (Miyamoto et al., 1996). However, Western blot analyses of integrins immunoprecipitated from MCF12A whole cell lysates did not detect an association with EGF receptors (EGFR). EGFR were readily detected in the MCF12A whole cell lysate at levels significantly greater than that of the normal HMEC lysate (Fig 2A, 1998 annual report). Immunofluorescece analyses of clustered integrins were inconclusive with regards to association of EGFR or other growth factor receptors (data not shown).

Since the EGF-receptor (EGFR) is overexpressed in MCF12A cells (Fig 2A, 1998 annual report), we examined the activation (tyrosine phosphorylation) of EGFR upon clustering of β1 integrins. Refeeding growth factor deprived MCF12A cells with media containing the normal (i.e. 100%) level of growth factors resulted in tyrosine phosphorylation of EGFR within 30 minutes. The addition of 1% GF alone stimulated partial activation of the EGFR. Cross-linking β1 integrins in the presence of 1% GF conditions did not increase EGFR tyrosine phosphorylation (Fig 3). Clustering of β1 integrins also did not appear to significantly affect the major pattern of tyrosine phosphorylation in whole cell lysates (Fig 4).
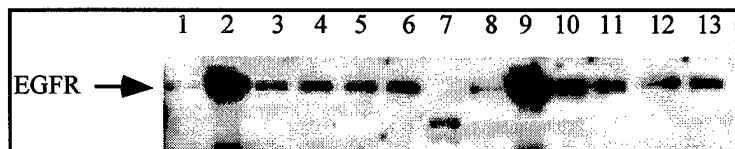

Fig 3. Tyrosine phosphorylation of EGFR following β1 integrin clustering on MCF12A cells. EGFR was immunoprecipitated from cell lysates prepared 30 min (lanes 1-6) or 60 min (lanes 8-13) after clustering of β1 integrins in 1% GF media. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to Immobilon membrane, incubated with anti-phosphotyrosine-HRP, and developed using ECL reagent. Cells were incubated in media with 0% GF alone (lanes 1,8); media with 100% GF alone (lanes 2,9); media with 1% GF alone (lanes 3, 10); mouse IgG1 + goat anti-mouse (lanes 4, 11); P4C10 (anti-β1)+ goat anti-mouse (lanes 5, 12); P4C10 alone (lanes 6, 13); MW standards (lane 7).
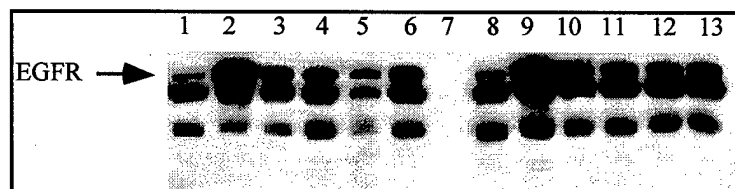

Fig 4. Tyrosine phosphorylation in whole cell lysates following β1 integrin clustering on MCF12A cells. Whole cell lysates prepared 30 min (lanes 1-6) or 60 min (lanes 8-13) after clustering of β1 integrins in 1% GF media were separated by SDS-PAGE, transferred to Immobilon membrane, incubated with anti-phosphotyrosine-HRP, and developed using ECL reagent. Cells were incubated in media with 0% GF alone (lanes 1,8); media with 100% GF alone (lanes 2,9); media with 1% GF alone (lanes 3, 10); mouse IgG1 + goat anti-mouse (lanes 4, 11); P4C10 + goat anti-mouse (lanes 5, 12); P4C10 alone (lanes 6, 13); MW standards (lane 7).

Integrins appear to activate the Ras-extracellular signal-regulated kinase (ERK) MAPK cascade through a couple different pathways (Giancotti and Ruoslahti, 1999). Activation of MAPK was examined using polyclonal rabbit antibodies specific for activated MAPK and for total MAPK. There was no change in MAPK (either p44 or p42) activation at 30 min or 60 min following clustering of β1 integrins. There also did not appear to be

activation of either focal adhesion kinase (FAK) or integrin linked kinase (ILK) in these β1 integrin-cluster lysates (data not shown).

Aim 3: Examine carcinoma cells for possible defects in these mechanisms.

Since we were unable to determine the integrin signaling mechanisms utilized by normal HMEC, we did not examine potential integrin signaling pathways in breast cancer cells.

However, during the course of analyzing affects on cell cycle components, we found that cyclin D2 was expressed by normal HMEC but not in the MCF12A or MDA-MB-435 cell lines (Fig 4 and Fig 5, 1998 annual report). Others have also reported a lack of cyclin D2 expression in breast cancer cell lines. Although cyclins D1, D2, and D3 each appear capable of mediating progression through the G1 restriction point of the cell cycle, they also appear to carry out distinct functions. Expression of cyclin D2 has been postulated to be involved in the maintenance of growth-arrest in fibroblasts (Meyyappan et al., 1998). and the differentiation of certain neural cell populations (Ross et al., 1996; Ross and Risken, 1994). In the study of mouse mammary tumorigenesis, Said et al (Said et al., 1995) suggested that cyclin D2 binding to cdk4 in hyperplasias may be an attempt to maintain or induce differentiation of the mammary epithelial cells. To evaluate the role of cyclin D2 expression in the regulation of cell proliferation and/or differentiation of mammary epithelial cells, we attempted to clone cyclin D2 from normal mammary epithelial cells and overexpress the gene in breast cancer cells. Cyclin D2 was successfully cloned from normal human mammary epithelial cells (HMEC) by RT-PCR. Unfortunately, cloning of cyclin D2 took much longer than anticipated due to the fact that the original 5' oligo primer failed to produce a pcr product. Valuable time was lost attempting to optimize other parameters of the PCR reaction and trying different HMEC cultures. Redesign of the 5' oligo primer to an overlapping region slightly downstream immediately produced a PCR product of the expected size (about 1.0 kb) as well as a smaller product of about 0.6 kb. Cloning and sequencing of the 1.0 kb material revealed one product, which was identical to the published sequence for human cyclin D2, and a second product that differed at a single nucleotide site. This single nucleotide change should not alter the corresponding amino acid. Cyclin D2 was cloned into the pcDNA 3.1(+) expression vector (Invitrogen, San Diego, CA). Two clones were isolated. One with cyclin D2 in the sense orientation (clone #13) and one clone in the anti-sense orientation (clone # 32). Transfection of MDA-MB-435 cells with clone #13, but not clone #32 nor pcDNA 3.1, produced a protein product of the expected size that reacted with a monoclonal antibody to cyclin D2 (Fig 5).
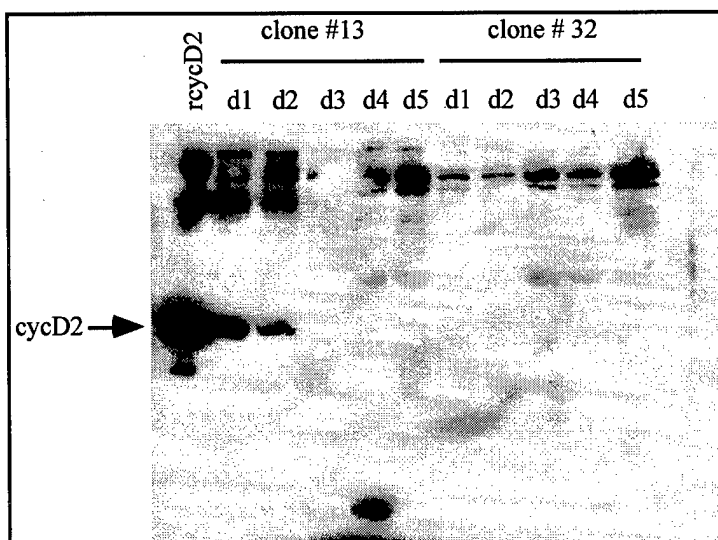
Fig 5. Transient expression of cyclin D2 in MDA-MB-435. MDA-MB-435 cells were transfected with either the sense cyclin D2 construct (clone #13) or the anti-sense cyclin D2 construct (clone #32). Whole cell lysates were prepared on days 1, 2, 3, 4, and 5 following transfection. One hundred micrograms were precipitated, resuspended in sample buffer, separated by SDS-PAGE, transferred to Immobilon-P, incubated with anti-cyclin D2 (LabVision, Fremont, CA), and developed with an ECL detection system. Recombinant cyclin D2 (rcycD2) expressed in baculovirus (Pharmingen, San Diego, CA) was included as a positive control.

Expression was greatest within the first 24 hours and was lost by 72 h. These results suggest that cyclin D2 protein is expressed from the cloned cDNA and that the protein is stable for less than 72 h. Analysis of transient transfection experiments was hampered by a bad lot of antibody from Pharmingen. Although the Pharmingen antibody had given excellent results in our initial analyses of HMEC and cell lines, it failed to perform reliably with cell lysates during this past year. Once again, valuable time was lost comparing different lysis buffers, starting new cell cultures, and checking protein determination and protein precipitation methods. This problem was solved by the purchase of a monoclonal antibody from LabVision (Fremont, CA).

Originally, we planned to isolate stable transfectants to determine the long-term effects of cyclin D2 expression on MDA-MB-435 cells (i.e. effects on cell growth, growth in Matrigel, expression of differentiation markers, and integrin modulation of cell proliferation). However, due to the technical difficulties encountered with this project and the budget constraints of this last year that limited technical help to one, half-time technician, we were only able to conduct the transient expression experiments. In an attempt to evaluate possible effects of cyclin D2 transient expression on differentiation, mRNA was analyzed for changes in expression of E-cadherin and the integrin $\alpha6A/\alpha6B$. E-cadherin expression was not induced nor was there any detectable change in the pattern of $\alpha6A/\alpha6B$ expression (data not shown).

Cyclin D2 expression was examined in a number of human breast cancer cell lines as well as HMEC and MCF12A cells by RT-PCR. Cyclin D2 was expressed in HMEC and in MCF12A, but at a much lower level (Fig 6). The predominant pcr product from the cyclin D2 reactions in breast cancer cell lines was the 0.6 kb pcr product (Fig 7). The 0.6 kb pcr product was cloned and sequenced. This 0.6 kb product does not have any

12

homology to cyclin D2 or other cyclin D genes. A BLAST search also did not show any significant homology to genes contained in the GenBank data base. The identity and significance of the 0.6 kb product remain unclear.
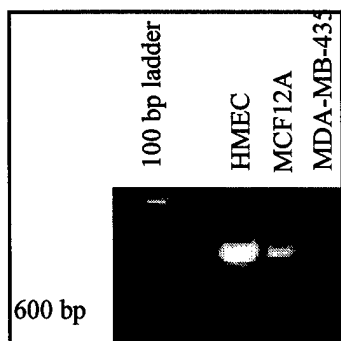

Fig 6. Cyclin D2 expression. Cyclin D2 expression in HMEC, MCF12A and MDA-MB-435 cells was assessed by RT-PCR.

1. 100 bp ladder
2. BC96.1
3. Hs578t
4. MDA-MB-361
5. HBL 100
6. HMEC
7. blank
8. 100 bp ladder

9. BT474
10. BT20
11. MCF7
12. MDA-MB-134V
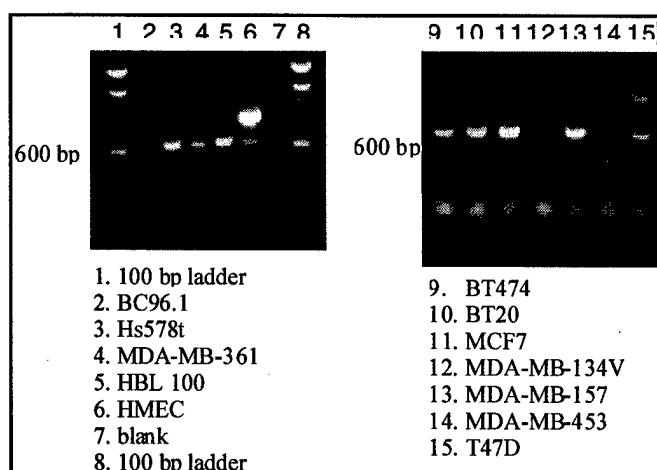13. MDA-MB-157
14. MDA-MB-453
15. T47D

Fig 7. Cyclin D2 expression in breast cancer cell lines. Cyclin D2 expression in breast cancer cell lines and in primary cells isolated from a tumor (BC96.1) was analyzed by RT-PCR.

Expression of integrin α6A and α6B. The integrin α6 exists as two alternatively spliced forms (α6BAand α6B) which differ in their cytoplasmic domains (Tamura et al., 1991; Cooper et al., 1991; Hogervorst et al., 1991). In general, the α6A isoform is expressed by normal epithelial cells, such as epidermal keratinocytes while α6B is expressed by most carcinomas and tends to be the predominant form expressed by teratocarcinoma cell lines and totipotent or pluripotent cells such as mouse embryonic stem cells. The expression of α6B may have a direct role in epidermal tumorigenesis (Tennenbaum et al., 1995). Analyses by RT-PCR revealed that normal HMEC and the normal mammary epithelial cell line MCF12A expressed only the α6A isoform while the highly metastatic mammary carcinoma cell line MDA-MB-435 expressed both α6A and α6B (Fig 6, 1998 update report). Analysis of RNA isolated from frozen sections of normal breast, in situ/invasive ductal

carcinoma, invasive carcinoma, and a lymph node metastasis demonstrated that invasive carcinoma and the lymph node metastasis expressed both isoforms of the integrin α6 (Fig 7, 1998 update report). These studies were expanded to a number of breast cancer cell lines and to 2 human breast cancer specimens (BC96.1 and BC97.1). All of the breast cancer cells expressed both α6A and α6B (Fig 8) while normal HMEC expressed only α6A. Two partially transformed cell lines, T47D (agar growth, non-tumorigenic in nude mice) and MDA-MB-453 (non-tumorigenic in nude mice) appeared to express primarily α6A. These results suggest that the expression of α6B may be a marker for breast cancer cells and possibly involved in tumorigenesis.

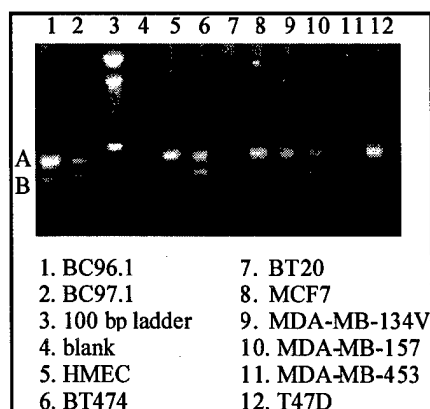| 1. BC96.1 | 7. BT20 |
2. BC97.1	8. MCF7
3. 100 bp ladder	9. MDA-MB-134V
4. blank	10. MDA-MB-157
5. HMEC	11. MDA-MB-453
6. BT474	12. T47D

Fig 8. Expression of integrin alpha 6A and alpha 6B. Expression of alternatively spliced forms of integrin alpha 6 (α6A and α6B) in various breast cancer cell lines and from 2 breast cancer specimens (BC96.1, BC97.1) were evaluated by RT-PCR.

KEY RESEARCH ACCOMPLISHMENTS

- Expanded the original GRGDS studies to normal HMEC and a number of breast carcinoma cell lines.
- Although α5β1 appears to modulate cell proliferation in fibroblasts and other non-epithelial cells, it does not appear to play a significant role in normal HMEC nor breast cancer cells.
- The primary integrins involved in promoting cell proliferation of normal HMEC appear to be α3β1 and α6β4. Other integrins may also affect components of the cell proliferation machinery.
- These integrin modulation mechanisms may be defective in metastatic breast cancer cells
- Cyclin D2 is expressed by normal HMEC but not by breast carcinoma cell lines.
- Integrin α6B is expressed by breast carcinoma cell lines but not by normal HMEC. This gene may be a marker for breast cancer tumorigenesis.

REPORTABLE OUTCOMES

Tamura, R.N., Pabich, W., Symington, B., and Wayner, E. (1997) Role of integrins in regulating cell proliferation of normal and neoplastic mammary epithelial cells. Era of Hope, The Department of Defense Breast Cancer Research Program Meeting, October 31, 1997.

CONCLUSIONS

The cell growth assay using antibody coated plates provided the most consistent and compelling evidence for integrin-mediated regulation of cell proliferation. Results from this assay suggest that integrin regulation occurs in normal mammary epithelial cells (MCF12A) but perhaps not in metastatic mammary epithelial cells (MDA-MB-435). The strongest effects occurred with antibodies to the $\beta1$, $\alpha3$, $\beta4$ and $\alpha6$ integrin subunits. These results strongly suggest that integrins $\alpha3\beta1$ and $\alpha6\beta4$ may be the primary integrins involved in potentiating cell proliferation in reduced growth factor conditions. This would be consistent with the results of others that $\beta4$ integrins promoted the proliferation of normal epidermal keratinocytes (Mainiero et al., 1997) and that $\alpha3\beta1$-laminin-5 interactions promoted the growth of mammary epithelial cells (Gonzales et al., 1999). The binding of $\alpha6\beta4$ to laminin-5 mediates the formation of hemidesmosomes which is the major anchoring structure for epithelial cells to the underlying stroma. Thus, interactions with laminin-5 through the integrins $\alpha3\beta1$ and $\alpha6\beta4$ may be important for normal mammary epithelial cell proliferation and homeostasis. Laminin-5 was readily detected in the basement membrane of ducts and tubules in both normal and cancer breast tissue but not around infiltrating cancer cells via immunohistochemistry. Infiltrating breast cancer cells expressed the integrin $\alpha3\beta1$ but not $\alpha6\beta4$. The integrin $\alpha3\beta1$ is also a receptor for type I collagen and fibronectin. Thus, infiltrating tumor cells may utilize the integrin $\alpha3\beta1$ to interact with type I collagen and fibronectin in the stroma. It is possible that loss of $\alpha6\beta4$ expression, or defects in $\alpha6\beta4$ function, while maintaining normal $\alpha3\beta1$ function promotes hyperplasia of mammary epithelial cells and tumorigenesis.

The signaling mechanism(s) involved in the integrin potentiation of growth-factor mediated cell proliferation remain unresolved. Although Integrin clustering appeared to promote Rb phosphorylation, it did not appear to affect expression of cyclin Ds, the association of EGFR with integrins, nor activation of EGFR, MAPK, FAK or ILK. It may be that the integrin clustering experiments did not closely mimic the antibody cell proliferation assays. Although the conditions used were sufficient to promote integrin clustering when evaluated by immunofluoresence staining of cells in chamber slides, it is possible that these conditions were ineffective when scaled up to 6 well multiwell plates or insufficient to activate signaling pathways. It may also be that these parameters were not analyzed at the proper time. For example, Gonzalez et al (Gonzales et al., 1999) observed a decrease in MAPK activation 48 h after the addition of monoclonal antibodies that perturb cell interactions with laminin-5. It may be that the addition of 1% growth factors to cells synchronized by complete growth factor deprivation is sufficient to stimulate progression through one round of the cell cycle but integrin clustering promotes an additional transit through the cell cycle.

The reasons for the lack of integrin modulation of cell proliferation in MDA-MB-435 cells are unknown. Several factors are likely to be involved. There appears to be changes in the growth-factor mechanisms that

permit MDA-MB-435 cells to proliferate in the presence of little exogenously added growth factors (fetal bovine serum). Synchronization of MDA-MB-435 cells required growth in completely serum-free conditions for about 7 days. This ability to proliferate in the absence of serum is reflected by the maintenance of the hyperphosphorylated state of Rb protein for 5 days after switching to completely serum-free media (data not shown). One factor that may contribute to the resistance of MDA-MB-435 cells to growth arrest by serum deprivation is the lack of expression of the cyclin dependent kinase inhibitor, p21 (data not shown).

Our data on the expression of cyclin D2 in breast cancer cell lines is consistent with the results of others for MCF-7, ZR-75, T-47D, HBL-100, and MCF-10A cell lines (Sweeney et al., 1997; Zhou et al., 1997). Buckley et al (Buckley et al., 1993) also reported that the expression of cyclin D2 was lower in breast cancer cell lines than in cultures of normal breast epithelial cells. Cyclin D1, D2 and D3 are a family of related proteins involved in regulation of progression through the G1 phase of the cell cycle. However, several differences have been reported among the cyclin D(s) for their binding to specific cdk(s), the formation of active or inactive kinase complexes, and binding to Rb protein (Ewen et al., 1993; Higashi et al., 1996; Kato et al., 1993; Sweeney et al., 1997; Tam et al., 1994). In the study of mouse mammary tumorigenesis, Said et al (Said et al., 1995) suggested that cyclin D2 binding to cdk4 in hyperplasias may be an attempt to maintain or induce differentiation of the mammary epithelial cells. Further investigation into the functions of cyclin D2 in normal human mammary epithelial cells appears warranted.

Another change in gene expression during breast cancer tumorigenesis may be with the expression of the integrin α6B isoform. Normal HMEC and the normal mammary epithelial cell line MCF12A express only the α6A isoform. The partially transformed carcinoma cell lines, T47D and MDA-MB-453 appear to express primarily the α6A isoform. All other breast carcinoma cell lines as well as RNA isolated from breast cancer tissue and tissue sections express significant amounts of the α6B mRNA. These results suggest that the expression of the α6B mRNA isoform may correlate with tumorigenesis and may provide a useful marker for the evaluation of clinical samples.

REFERENCES

Buckley, M. F., Sweeney, K. J., Hamilton, J. A., Sini, R. L., Manning, D. L., Nicholson, R. I., deFazio, A., Watts, C. K., Musgrove, E. A., and Sutherland, R. L. (1993). Expression and amplification of cyclin genes in human breast cancer. Oncogene *8*, 2127-33.

Clark, E. A., and Brugge, J. S. (1995). Integrins and signal transduction pathways: the road taken. Science *268*, 233-9.

Cooper, H. M., Tamura, R. N., and Quaranta, V. (1991). The major laminin receptor of mouse embryonic stem cells is a novel isoform of the alpha 6 beta 1 integrin. J Cell Biol *115*, 843-50.

Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J., and Livingston, D. M. (1993). Functional interactions of the retinoblastoma protein with mammalian D- type cyclins. Cell *73*, 487-97.

Giancotti, F. G., and Ruoslahti, E. (1999). Integrin signaling. Science *285*, 1028-32.

Gonzales, M., Haan, K., Baker, S. E., Fitchmun, M., Todorov, I., Weitzman, S., and Jones, J. C. (1999). A cell signal pathway involving laminin-5, alpha3beta1 integrin, and mitogen-activated protein kinase can regulate epithelial cell proliferation. Mol Biol Cell *10*, 259-70.

Higashi, H., Suzuki-Takahashi, I., Saitoh, S., Segawa, K., Taya, Y., Okuyama, A., Nishimura, S., and Kitagawa, M. (1996). Cyclin-dependent kinase-2 (Cdk2) forms an inactive complex with cyclin D1 since Cdk2 associated with cyclin D1 is not phosphorylated by Cdk7- cyclin-H. Eur J Biochem *237*, 460-7.

Hogervorst, F., Kuikman, I., van Kessel, A. G., and Sonnenberg, A. (1991). Molecular cloning of the human alpha 6 integrin subunit. Alternative splicing of alpha 6 mRNA and chromosomal localization of the alpha 6 and beta 4 genes. Eur J Biochem *199*, 425-33.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11-25.

Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev *7*, 331-42.

Mainiero, F., Murgia, C., Wary, K. K., Curatola, A. M., Pepe, A., Blumemberg, M., Westwick, J. K., Der, C. J., and Giancotti, F. G. (1997). The coupling of alpha6beta4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. Embo J *16*, 2365-75.

Meyyappan, M., Wong, H., Hull, C., and Riabowol, K. T. (1998). Increased expression of cyclin D2 during multiple states of growth arrest in primary and established cells. Mol Cell Biol *18*, 3163-72.

Miyamoto, S., Teramoto, H., Gutkind, J. S., and Yamada, K. M. (1996). Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. J Cell Biol *135*, 1633-42.

Plopper, G. E., McNamee, H. P., Dike, L. E., Bojanowski, K., and Ingber, D. E. (1995). Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol Biol Cell *6*, 1349-65.

Ross, M. E., Carter, M. L., and Lee, J. H. (1996). MN20, a D2 cyclin, is transiently expressed in selected neural populations during embryogenesis. J Neurosci *16*, 210-9.

Ross, M. E., and Risken, M. (1994). MN20, a D2 cyclin found in brain, is implicated in neural differentiation. J Neurosci *14*, 6384-91.

Said, T. K., Luo, L., and Medina, D. (1995). Mouse mammary hyperplasias and neoplasias exhibit different patterns of cyclins D1 and D2 binding to cdk4. Carcinogenesis *16*, 2507-13.

Sweeney, K. J., Sarcevic, B., Sutherland, R. L., and Musgrove, E. A. (1997). Cyclin D2 activates Cdk2 in preference to Cdk4 in human breast epithelial cells. Oncogene *14*, 1329-40.

Symington, B. E. (1992). Fibronectin receptor modulates cyclin-dependent kinase activity. J Biol Chem *267*, 25744-7.

Symington, B. E. (1995). Growth signalling through the alpha 5 beta 1 fibronectin receptor. Biochem Biophys Res Commun *208*, 126-34.

Tam, S. W., Theodoras, A. M., Shay, J. W., Draetta, G. F., and Pagano, M. (1994). Differential expression and regulation of Cyclin D1 protein in normal and tumor human cells: association with Cdk4 is required for Cyclin D1 function in G1 progression. Oncogene *9*, 2663-74.

Tamura, R. N., Cooper, H. M., Collo, G., and Quaranta, V. (1991). Cell type-specific integrin variants with alternative alpha chain cytoplasmic domains. Proc Natl Acad Sci U S A *88*, 10183-7.

Tennenbaum, T., Belanger, A. J., Glick, A. B., Tamura, R., Quaranta, V., and Yuspa, S. H. (1995). A splice variant of alpha 6 integrin is associated with malignant conversion in mouse skin tumorigenesis. Proc Natl Acad Sci U S A *92*, 7041-5.

Zhou, Q., Stetler-Stevenson, M., and Steeg, P. S. (1997). Inhibition of cyclin D expression in human breast carcinoma cells by retinoids in vitro. Oncogene *15*, 107-15.

Appendix

A. Bibliography of publication and meeting abstracts.

   Publications : None

   Abstracts:

   Tamura, R.N., Pabich, W., Symington, B., and Wayner, E. (1997) Role of integrins in regulating cell proliferation of normal and neoplastic mammary epithelial cells. Era of Hope, The Department of Defense Breast Cancer Research Program Meeting, October 31, 1997.

B. List of Personnel (1994-1999)

   1. Elizabeth Wayner, Ph.D. Principal Investigator
   2. Richard Tamura, Ph. D. co-Principal Investigator
   3. Wendy Pabich, Research Technician I/II
   4. Irina Ankoudinova, Research Technician I
   5. Heidi Hummel, Graduate student
   6. John Fisher, Research Technician II

Role of Integrins in Regulating Cell Proliferation of Normal and Neoplastic Mammary Epithelial Cells

Richard N. Tamura, Wendy Pabich, Banu Symington, and Elizabeth Wayner

ABSTRACT

The extracellular matrix (ECM) is a milieu of proteins that provides structural integrity to tissues and organs as well as cues regulating several cell functions such as proliferation, migration, differentiation, and gene expression. These cell-ECM interactions are mediated, in part, through the integrin family of cell adhesion receptors.

We have been investigating the role of integrins in the regulation of cell cycle progression. Initial experiments suggested that peptides containing the cell adhesion motif of Arg-Gly-Asp (RGD) regulated cdc2 kinase/cyclin A associations via the integrin $\alpha5\beta1$. Regulation was observed in normal human epidermal keratinocytes and the non-tumorigenic breast cancer cell line HBL 100 but not in the tumorigenic cell line BT20 or virally-transformed keratinocytes. These results suggested a potential defect in the $\alpha5\beta1$ signaling pathway regulating cell cycle progression in transformed cells. We extended these studies to normal mammary epithelial cells (HMEC) and to a number of additional breast cancer cell lines (T47D, DU4475, MDA-MB-453, MDA-MB-157, BT 483, Hs578t, MDA-MB-361, MCF7, BT474, and MDA-MB-134-IV) which vary from being partially transformed (agar growth/non-tumorigenic) to fully transformed (tumorigenic/metastatic). In order to understand the interaction of the RGD peptides with these various cell lines and HMEC, we characterized the repertoire of integrin expression by FACS. Of the three integrins that bind soluble RGD peptides with the highest avidity, $\alpha v\beta5$ was expressed by nearly all of the cell lines, 40% expressed $\alpha v\beta3$, and 60% expressed $\alpha5\beta1$. HMEC expressed intermediate levels of $\alpha5$ and $\alpha4$, low levels of $\alpha6$ and $\alpha v\beta5$, and were negative for $\alpha v\beta3$. The most prominent integrin expressed by HMEC and by all of the cell lines was $\alpha3\beta1$, which is primarily a receptor for laminins but also mediates adhesion to fibronectin and collagens.

Incubation with RGD peptides resulted in increases in both cdc2 kinase activity and cyclin A associated kinase activity in HMEC and a number of the cell lines. However, in most cases, the stimulation of cdc2 kinase activity or cyclin A associated kinase activity appeared to be mutually exclusive suggesting a mechanism that is distinct from that of normal human epidermal keratinocytes or HBL100 cells. There was also no apparent correlation between the stimulation of cell cycle kinases with either tumorigenic potential or expression of the integrins $\alpha5\beta1$, $\alpha v\beta3$, or $\alpha v\beta5$.

Immunohistochemical analysis of normal and tumor breast tissue indicated that $\alpha5\beta1$ was not expressed by either normal mammary epithelial cells or tumor cells. Basal mammary epithelial cells expressed integrins $\alpha3$, $\alpha6$, $\beta4$, $\alpha2$, $\beta1$, $\alpha v\beta5$, and possibly $\alpha v\beta6$, luminal cells and cancer cells (in situ and invasive ductal) expressed predominately $\alpha3\beta1$. A few samples were positive for $\alpha v\beta3$ in the basal cells. The integrin $\alpha4\beta1$ was not expressed by either normal epithelial cells or cancer cells. These results suggest that $\alpha5\beta1$ is not of primary importance in mediating cell adhesive signaling events in either normal mammary epithelial cell or during tumorigenesis.

Recently, we have initiated studies to examine cell proliferation regulation mediated through specific integrins by clustering with monoclonal antibodies. Preliminary results suggest that a number of integrins, $\beta 1$, αv, $\alpha 3$, and $\beta 4$, may promote transit of normal HMEC through the cell cycle under reduced serum conditions as evaluated by the time course of retinoblastoma (Rb) phosphorylation. Similar results have been demonstrated by others for $\beta 1$ integrins in fibroblast cells and is thought to involve clustering of growth factor receptors and activation of mitogen activated protein kinase (MAPK). We are looking to see if similar mechanisms are being used by normal mammary epithelial cells, both basal and luminal, and breast cancer cells. Understanding the role of integrins in the regulation of cell proliferation may lead to the development of novel therapeutics for the treatment of breast cancer.